Journal of Cellular Physiology

Evaluation of Antioxiodant Drugs for the Treatment of Ochronotic Alkaptonuria in An *In Vitro* Human Cell Model

LAURA TINTI,[1] ADRIANO SPREAFICO,[1,2]** DANIELA BRACONI,[3] LIA MILLUCCI,[3] GIULIA BERNARDINI,[3] FEDERICO CHELLINI,[1] GIOVANNI CAVALLO,[2] ENRICO SELVI,[1,2] MAURO GALEAZZI,[2,3] ROBERTO MARCOLONGO,[2] JAMES A. GALLAGHER,[2,4] AND ANNALISA SANTUCCI[2,3]*

[1]*Sezione di Reumatologia, Dipartimento di Medicina Clinica e Scienze Immunologiche, Università degli Studi di Siena, Policlinico Le Scotte, Siena, Italy*

[2]*Centro Interdipartimentale per lo Studio Biochimico delle Patologie Osteoarticolari, Università degli Studi di Siena, Siena, Italy*

[3]*Dipartimento di Biologia Molecolare, Università degli Studi di Siena, Siena, Italy*

[4]*Department of Human Anatomy & Cell Biology, University of Liverpool, Liverpool, UK*

Alkaptonuria (AKU) is a rare autosomal recessive disease, associated with deficiency of homogentisate 1,2-dioxygenase activity in the liver. This leads to an accumulation of homogentisic acid (HGA) and its oxidized derivatives in polymerized form in connective tissues especially in joints. Currently, AKU lacks an appropriate therapy. Hence, we propose a new treatment for AKU using the antioxidant *N*-acetylcysteine (NAC) administered in combinations with ascorbic acid (ASC) since it has been proven that NAC counteracts the side-effects of ASC. We established an *in vitro* cell model using human articular primary chondrocytes challenged with an excess of HGA (0.33 mM). We used this experimental model to undertake pre-clinical testing of potential antioxidative therapies for AKU, evaluating apoptosis, viability, proliferation, and metabolism of chondrocytes exposed to HGA and treated with NAC and ASC administered alone or in combination addition of both. NAC decreased apoptosis induced in chondrocytes by HGA, increased chondrocyte growth reduced by HGA, and partially restored proteoglycan release inhibited by HGA. A significantly improvement in efficacy was found with combined addition of the two antioxidants in comparison with NAC and ASC alone. Our novel *in vitro* AKU model allowed us to demonstrate the efficacy of the co-administration of NAC and ASC to counteract the negative effects of HGA for the treatment of ochronotic arthropathy.

J. Cell. Physiol. 225: 84–91, 2010.

Alkaptonuria (AKU) results from the deficiency of the enzyme homogentisate 1,2-dioxygenase (HGO). Homogentisic acid (HGA) is a product of tyrosine and phenylalanine catabolism (La Du et al., 1958; Fernández-Cañón et al., 1996; La Du, 2001). HGA is excreted in the urine, and imparts a black discoloration upon oxidation (Zannoni et al., 1962). However, in urine and in tissues, HGA can be oxidized to benzoquinone acetic acid (BQA), which forms melanin-like polymers (O'Brien et al., 1963; La Du, 2002), which are deposited in the connective tissue, most commonly the joints, but also in the cardiovascular system, kidneys, and skin (O'Brien et al., 1963; Selvi et al., 2000; Phornphutkul et al., 2002; Helliwell et al., 2008), causing a pathological pigmentation known as ochronosis (Zannoni et al., 1962; Gaines, 1989). Deposition of ochronotic pigment is not uniform and can be intracellular and extracellular, the latter being closely associated with initial nucleation on collagen fibers (Taylor et al., 2010). Eventually, ochronosis leads to degeneration of cartilage, chronic inflammation, and osteoarthritis (Selvi et al., 2000; Helliwell et al., 2008).

AKU lacks an appropriate therapy. Dietary restriction of tyrosine and phenylalanine intake has met with a limited success owing to the difficulty in maintaining such a regimen for the whole of a person's life. Furthermore, much of the excreted HGA is derived from turnover of existing proteins. A trial of nitisinone, an inhibitor of 4-hydroxyphenylpyruvate oxidase, in AKU sufferers has been undertaken at NIH (Suwannarat et al., 2005). However, in some cases plasma tyrosine levels rose significantly causing corneal damage and type III tyrosinemia (Keller et al., 2005). Ascorbic acid (ASC) was adopted to prevent HGA oxidation to BQA and to prevent reactive oxygen species (ROS) formation (Kutty et al., 1974). Unfortunately, therapy with ASC decreased the urinary HGA levels in some cases and ASC may also auto-co-oxidate with HGA to produce additional ROS (Martin and Batkoff, 1987).

N-acetylcysteine (NAC) is an antioxidant already used therapeutically as a mucolytic agent in respiratory conditions and in the management of paracetamol toxicity. NAC works as

Laura Tinti and Adriano Spreafico contributed equally to this study.

Contract grant sponsor: Fondazione Monte dei Paschi di Siena Esercizio 2008 and 2009.
Contract grant sponsor: Toscana Life Sciences Foundation Orphan 1;
Contract grant number: 0108.

*Correspondence to: Adriano Spreafico, Dipartimento di Medicina Clinica e Scienze Immunologiche, Università degli Studi di Siena, Sezione di Reumatologia, Policlinico Le Scotte, 53100 Siena, Italy. E-mail: spreafico@unisi.it

**Correspondence to: Annalisa Santucci, Dipartimento di Biologia Molecolare, Università degli Studi di Siena, via Fiorentina 1, 53100 Siena, Italy. E-mail: santucci@unisi.it

Received 10 February 2010; Accepted 31 March 2010

Published online in Wiley InterScience (www.interscience.wiley.com.), 27 May 2010.
DOI: 10.1002/jcp.22199

an analog and precursor of intracellular L-cysteine and reduced glutathione. NAC safety is supported by more than 40 years of clinical use. A number of experimental studies in clinical trials provide evidence that suggests that NAC inhibit oxidative, genotoxic, and carcinogenic effects. We previously demonstrated that NAC inhibits HGA polymerization (Chindamo et al., 2003; Lorenzini et al., 2003; Mannoni et al., 2004). This antioxidant could interfere with AKU pathogenic mechanisms in three ways. Firstly, acting as a scavenger of ROS and thereby limiting tissue damage. Secondly, preventing or delaying HGA accumulation. Finally, since NAC can neutralize the acetaminophene derivative *N*-acetylbenzoquinoneimine, whose chemical structure is similar to that of BQA, NAC could directly neutralize BQA.

One of the major obstacles to progress in therapeutic strategies is the lack of *in vitro* and *in vivo* models to study ochronosis (Kirkpatrick et al., 1984; Angeles et al., 1989). Here, we introduce an *in vitro* cell model utilizing primary chondrocytes isolated from human articular cartilage and treated with an excess of HGA. The emergence of this experimental model has presented us with an opportunity to undertake pre-clinical testing of potential antioxidative therapies for AKU.

Using this human ochronotic cell model, we investigated the effects of NAC and ASC treatment on proliferation, apoptosis, protein carbonylation, and metabolism of chondrocytes exposed to HGA. We found that HGA induced apoptosis and strongly reduced proteoglycan (PG) synthesis in HGA-treated human articular chondrocytes. NAC treatment was able to restore viability, proliferation, and chondrocyte anabolism, especially when administered in combination with ASC.

We thus propose a combination of NAC and ASC as a novel pharmacological intervention for AKU treatment.

Materials and Methods

Isolation and culture of human articular chondrocytes *in vitro*

Human articular cartilage fragments were obtained from femoral heads, with informed consent of patients undergoing surgery for total hip replacement. The study received approval from the Local Ethics Committee. Immediately after surgery, chondrocytes were isolated from articular cartilage as described (Grigolo et al., 2002).

Treatments

First passage human articular chondrocytes were seeded into 24-well plates at a density of 4×10^4 cells/well and grown until nearly confluence. Medium was changed twice a week.

HGA treatment. Cells were treated with HGA (1, 0.33, and 0.1 mM) in DMEM supplemented with 10% FCS.

HGA and NAC co-treatment. Chondrocytes were treated for 7 days with a combination of NAC (10, 1, 0.1, and 0.01 μM) and HGA (0.33 mM).

NAC and ASC pre-treatment. Chondrocytes were pre-incubated in culture medium in the absence or presence of NAC (10–0.01 μM), ASC (10–0.1 μM) and the combination of NAC *plus* ASC. After 24 h, the medium was replaced with culture medium containing the same antioxidant concentrations used for pre-incubation and 0.33 mM HGA was added.

Apotosis assay

Apoptosis was evaluated after 48 h of treatment using flow cytometry for the assessment of Annexin V binding (DAKO; Vermes et al., 1995).

Viability assay

Viability of pre-confluent human primary articular chondrocytes was evaluated in monolayer cultures after 4, 7, and 14 days of treatment using the MTT assay (Sigma–Aldrich, Milan, Italy) and correlated to the number of living cells (Denizot and Lang, 1986).

Proliferation assay

The proliferative response was evaluated 72 h after the beginning of treatment. The medium was removed, cleared by centrifugation and stored at −80°C for later detection of PG release. DNA was extracted from each culture and fluorimetrically quantified by the Qubit™ system (Invitrogen, San Giuliano Milanese (MI), Italy).

Histological analyses

Human primary articular chondrocytes were cultured at 4×10^4 cells/well on sterile coverslips, washed in isotonic saline solution, fixed in paraformaldehyde (4%), stained with Schmorl's dye, air dried, and mounted on histological slides.

Proteoglycan (PGs) release assay

PG release was assayed by an immunoenzymatic method (BioSource, San Giuliano Milanese (MI), Italy) according to the instructions of the manufacturer. Results were normalized to the DNA content of the cultures.

Analysis of protein carbonylation

Cell cultures were washed twice with PBS and resuspended in 500 μl of lysis buffer, 8 M urea, 4% (w/v) CHAPS, 40 mM Tris, and 65 mM dithioerythritol. Cell disruption was achieved through 5 min sonication in an ice bath. Protein content was determined by the Bradford technique (Bradford, 1976).

Ten micrograms of protein were incubated in the dark in 6% (w/v) SDS, 5% (v/v) trifluoroacetic acid, and 5 mM 2,4-dinitrophenylhydrazine to derivatize carbonyls. The buffer reaction was neutralized with 2 M Tris containing 30% (v/v) glycerol and 2% (v/v) β-mercaptoethanol (Dalle-Donne et al., 2003). Samples were subjected to SDS–PAGE (12% polyacrylamide; Laemmli, 1970). Gels were stained with Coomassie blue (Candiano et al., 2004) or transferred to PVDF by semi-dry blotting. Membranes were incubated with rabbit anti-dinitrophenyl antibodies (Sigma–Aldrich) 1:10,000, followed by peroxidase-conjugated anti-rabbit antibodies (Sigma–Aldrich) 1:7,000, and revealed by chemiluminescence (Bio-Rad, Segrate (MI), Italy).

Images of gels and films were acquired by ImageScanner (GE-Healthcare, Milano, Italy) and analyzed with ImageMaster Platinum (GE-Healthcare), choosing as a reference parameter the intensity of bands, automatically normalized against the background.

Statistics

Each experiment was performed in triplicate. Data were expressed as the mean ± SD. Student's *t*-test and multiple-measurement ANOVA analysis followed by the Bonferroni-type multiple comparison were used. Differences with *P*-value <0.05 were considered significant.

Results

Induction of apoptosis by HGA in human articular chondrocytes

A long exposure to HGA may induce a cytotoxic effect (Kirkpatrick et al., 1984; Angeles et al., 1989). We investigated the cytotoxic effect of different concentrations of HGA in order to find out the optimal concentration leading to the formation of ochronotic pigment with minimal effects on cell viability. Apoptosis was chosen as a reference parameter to evaluate HGA cytotoxicity. Chondrocytes were incubated for 48 h with different concentrations of HGA (0.1, 0.33, and 1 mM). 0.1 mM HGA did not show a significant cytotoxic effect, while 0.33 mM HGA induced apoptosis in about 26% of cells and 1 mM HGA led to apoptosis in about 96% of cells (Fig. 1). Results were confirmed by histological analyses of chondrocytes challenged with 1, 0.33, and 0.1 mM HGA for 4 days and stained with Schmorl's dye for ochronotic pigmentation (Fig. 1B). 0.33 mM HGA led to the darkest observable pigmentation with respect

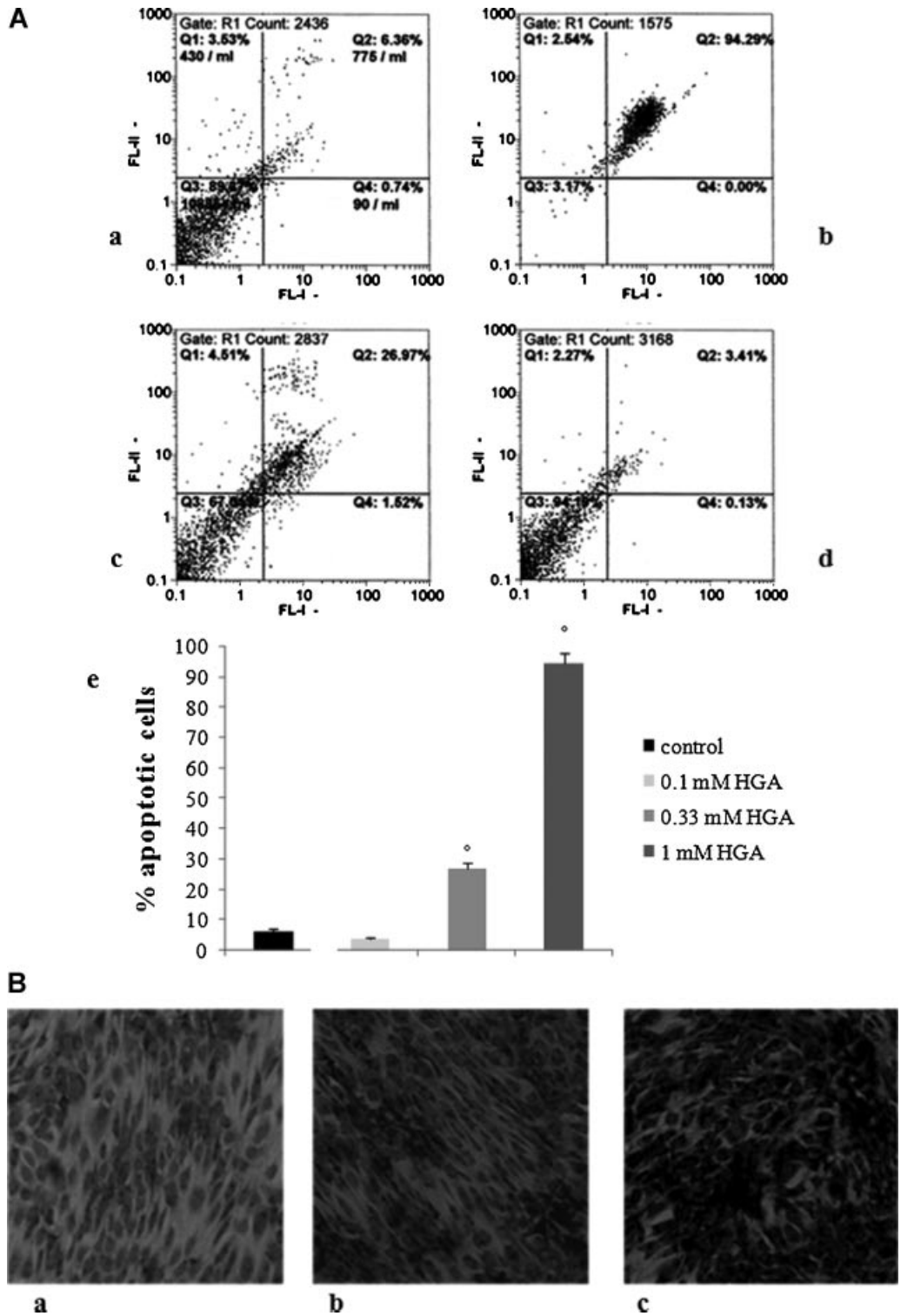

Fig. 1. A: Contour diagrams of FITC-Annexin V/PI flow cytometry of chondrocytes incubated with or without different concentrations of HGA, for 48 h. (a) Chondrocytes without HGA. (b) Chondrocytes treated with 1 mM HGA. (c) Chondrocytes treated with 0.33 mM HGA. (d) Chondrocytes treated with 0.1 mM HGA. The lower left quadrants (Q3) of each part show the viable cells, which exclude PI and were negative for FITC-Annexin V binding. The upper right quadrants (Q2) contain the non-viable, apoptotic cells, positive for FITC-Annexin V binding and for PI uptake. (e) Histogram representation of apoptotic chondrocytes after 48 h stimulation using different concentrations of HGA. B: (a) Control human articular chondrocytes after 4 days in subculture. Cells were stained using Schmorl's dye. Nucleus and cytoplasm are clearly distinguishable. (b) Human articular chondrocytes treated for the last 4 days of subculture with 0.1 mM HGA. (c) Human articular chondrocytes treated for 4 days of subculture with 0.33 mM HGA. Statistical significance compared to the control °$P < 0.05$.

to control (Fig. 1B-c), while 1 mM HGA exerted a major cytotoxic effect causing detachment of cells from coverslips. Therefore, 0.33 mM was chosen as the test HGA concentration in subsequent experiments.

Effects of antioxidants on viability, proliferation, and apoptosis of HGA-treated human articular chondrocytes

To assess the ability of NAC to counteract the effects of HGA, monolayer cultures of chondrocytes were pre-incubated with different NAC concentrations (0.01–10 μM) for 24 h followed by treatment with 0.33 mM HGA. In a parallel experiment, cultures were co-incubated with different NAC concentrations (0.01–10 μM) and 0.33 mM HGA. NAC administration reduced the presence of visible pigment in HGA-treated cells in a dose-dependent manner (Fig. 2A). Co-treatment with NAC and HGA was only effective with higher concentrations (1 and 10 μM). Moreover, while HGA administration significantly reduced chondrocyte proliferation, NAC pre-treatment effectively counteracted this reduction (Fig. 2B-a) and also

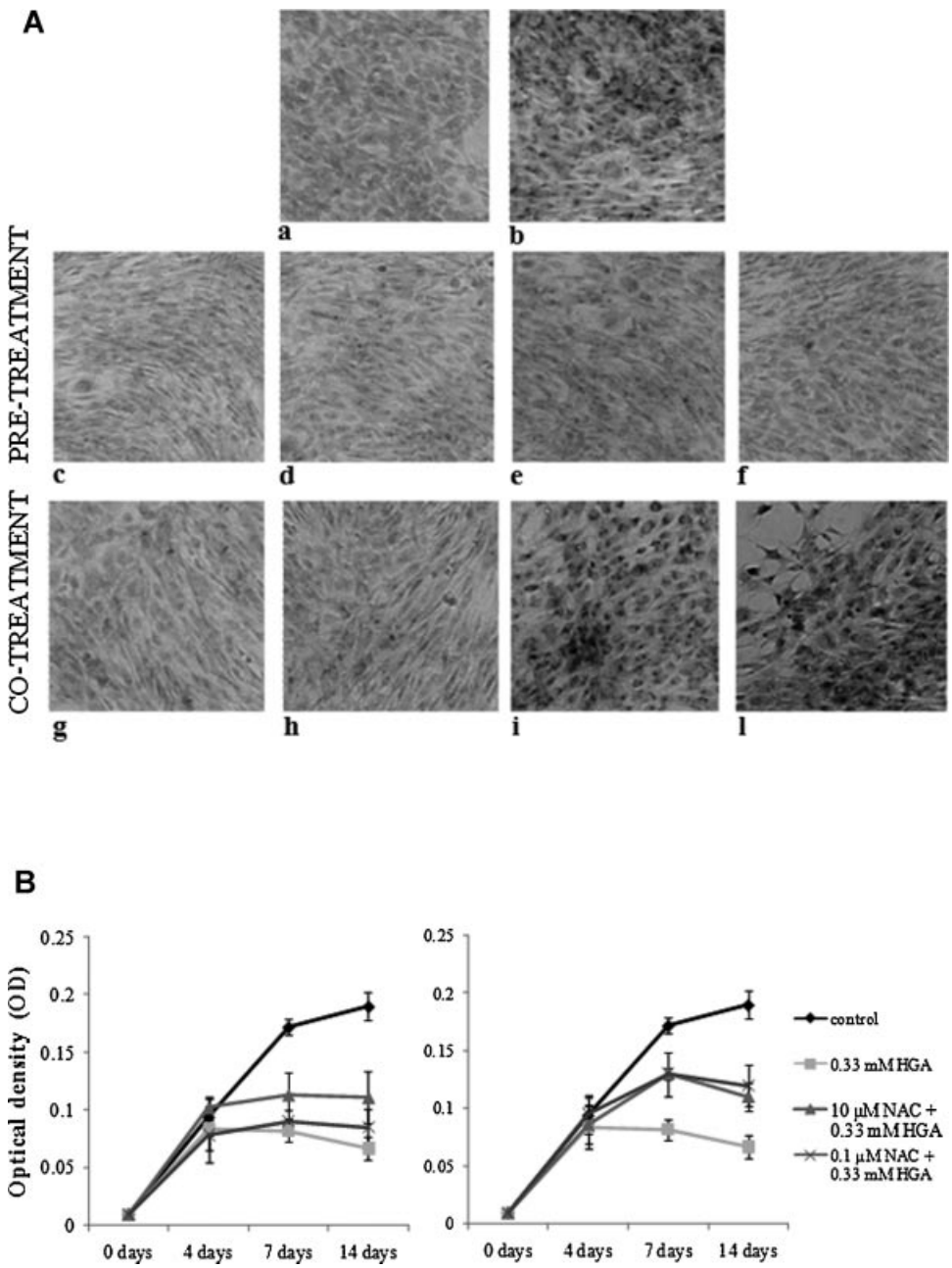

Fig. 2. A: Monolayer cultures of HGA-treated human articular chondrocytes pre-incubated with NAC. Cells were stained using Schmorl's dye. (a) Control human articular chondrocytes after 7 days in subculture. (b) Human articular chondrocytes treated for 7 days of subculture with 0.33 mM HGA. (c–f) Human articular chondrocytes *pre-incubated* with different concentrations of NAC: (c) 10 μM, (d) 1 μM, (e) 0.1 μM, (f) 0.01 μM. (g–l) Human articular chondrocytes *co-incubated* with different concentrations of NAC: (g) 10 μM, (h) 1 μM, (i) 0.1 μM, (l) 0.01 μM. B: Viability of HGA-treated human articular chondrocytes was evaluated after 0, 4, 7, and 14 days, using MTT test. (a) Cells were pre-treated for 24 h with different concentrations of NAC (10 and 0.1 μM) and then with HGA (0.33 mM) replacing the medium every 48 h. (b) Human articular chondrocytes were co-treated with different concentrations of NAC (10 and 0.1 μM) and then with HGA (0.33 mM) replacing the medium every 48 h.

reduced apoptosis (data not shown). However, simultaneous addition of NAC with HGA was much less effective in preventing pigmentation. Hence, we chose to adopt only pre-treatment with NAC in the subsequent experiments on the *in vitro* AKU cell models.

We investigated if pre-treatment with NAC or ASC or simultaneous addition of both could counteract the cytotoxic effect of HGA on chondrocytes. For both NAC and ASC, we used the two concentrations (0.1 and 10 μM) which were most effective in the experiments described above. HGA inhibited cell proliferation in comparison to control chondrocytes, reaching a maximum of 65% inhibition after 14 days of treatment. Each antioxidant at 0.1 mM restored chondrocyte proliferation, up to 76% at the end of the time course for the NAC + ASC treatment, up to 70% for ASC treatment and up to 65% for NAC treatment (Fig. 3A). Remarkably, after 14 days of treatment, when HGA exerted its strongest cytotoxic effect, antioxidants were still effective in counteracting it. We confirmed this observation by means of a DNA quantification assay (Fig. 3B). The administered antioxidants prevented the growth arrest associated with HGA exposure in a dose-dependent manner. NAC + ASC treatment had the greatest influence on cell growth, leading to an increase of the number of chondrocytes even in comparison to basal conditions.

In order to determine if the observed decrease in proliferation and viability in HGA-treated chondrocytes could be ascribed to HGA-induced apoptosis, we performed a flow cytometric analysis. Chondrocytes were pre-incubated for 24 h with 10 μM antioxidants followed by a further treatment with 0.33 mM HGA for 48 h. We found that HGA-induced apoptosis (+50% vs. control) and that 10 μM NAC was able to counteract HGA-induced apoptosis and also to prevent the pro-apoptotic effect of ASC (+139% vs. control), as shown in the challenge with both antioxidants in combination (Fig. 3C). Indeed, ASC may auto-co-oxidate together with HGA leading to the production of additional ROS (Martin and Batkoff, 1987; Montagutelli et al., 1994).

Oxidative carbonylation of proteins

Oxidative damage due to HGA, as well as effects of pre-treatments with ASC and NAC antioxidants, were evaluated through the analysis of protein carbonylation (Fig. 4). Overall, when cells were treated with HGA, or pre-treated with ASC before the addition of HGA, we found higher levels of carbonylated proteins than in the control cultures. The pre-treatment with NAC, alone or in combination with ASC, restored protein carbonyl levels to control values. Following quantitative addition of the intensities of immunoreactive bands in specific molecular weight ranges, we found: (i) the specific carbonylation of a band between 100 and 150 kDa in the presence of HGA, which was also found after pre-treating with ASC or NAC + ASC, but not found after pre-treating with NAC; (ii) the specific carbonylation of a band between 75 and 100 kDa in the presence of HGA, also found to a lesser extent after pre-treating cells with ASC, NAC, or NAC + ASC; (iii) a significant HGA-induced carbonylation (almost twofold greater than the control) of proteins ranging 25–50 kDa which were protected from carbonylation following pre-treatment with NAC and NAC + ASC.

Effects of antioxidants on proteoglycan release

PG synthesis, which is a biomarker of chondrocyte anabolism, was chosen as a reference parameter to evaluate the effects of HGA treatment of human articular chondrocyte metabolism and function. The cells were pre-treated with antioxidants for 24 h and challenged with 0.33 mM HGA for the next 48 h. HGA treatment led to a dramatic decrease of PG release (Fig. 5). Hence, we have highlighted the ability of 10 μM NAC, 10 μM ASC but especially the combined addition of both to partially restore PG synthesis (by 18%, 8%, and 27%, respectively).

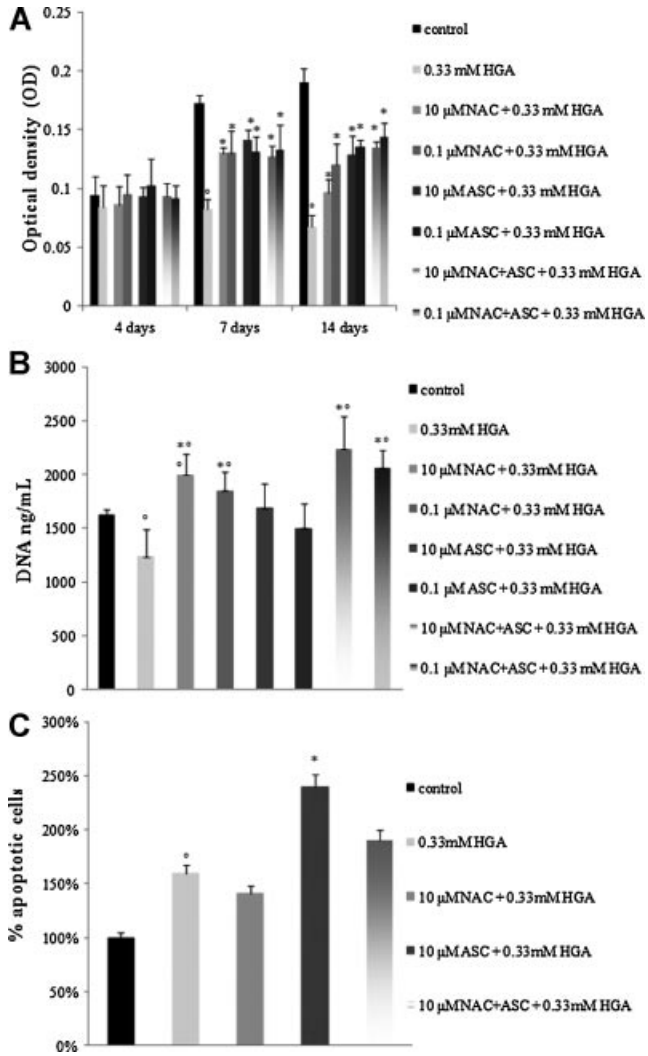

Fig. 3. A: MTT viability assay of HGA-treated human articular chondrocytes. Cells were pre-treated for 24 h NAC and/or ASC at different concentrations (10 and 0.1 μM) and then challenged with HGA (0.33 mM) replacing the medium every 48 h. Cells were assayed at 4–7 and 14 days of culture. B: Proliferation of HGA-treated human articular chondrocytes. Cells were pre-incubated, for 24 h, with NAC and/or ASC at different concentrations (10 and 0.1 μM) and then treated with HGA (0.33 mM) for 48 h. Proliferative response of chondrocytes was evaluated measuring their DNA content after 48 h of treatment with HGA. C: Apoptosis of HGA-treated human articular chondrocytes. Chondrocytes were pre-incubated for 24 h with NAC and/or ASC, at the concentration 10 μM and then challenged with HGA (0.33 mM) for 48 h. Apoptotic response of chondrocytes was measured at 48 h from the treatment with HGA using flow cytometry. The number of apoptotic cells in the different cultures is expressed in percentage considering as 100% the number of apoptotic cells in the control cultures. Statistical significance compared to the control ° $P < 0.05$. Statistical significance compared to 0.33 mM HGA *$P < 0.05$.

Discussion

Although AKU pathological features are clinically well described, the molecular basis of the disease has not been explored to any significant degree. Major obstacles to progress include the difficulty in obtaining human tissues and cells from

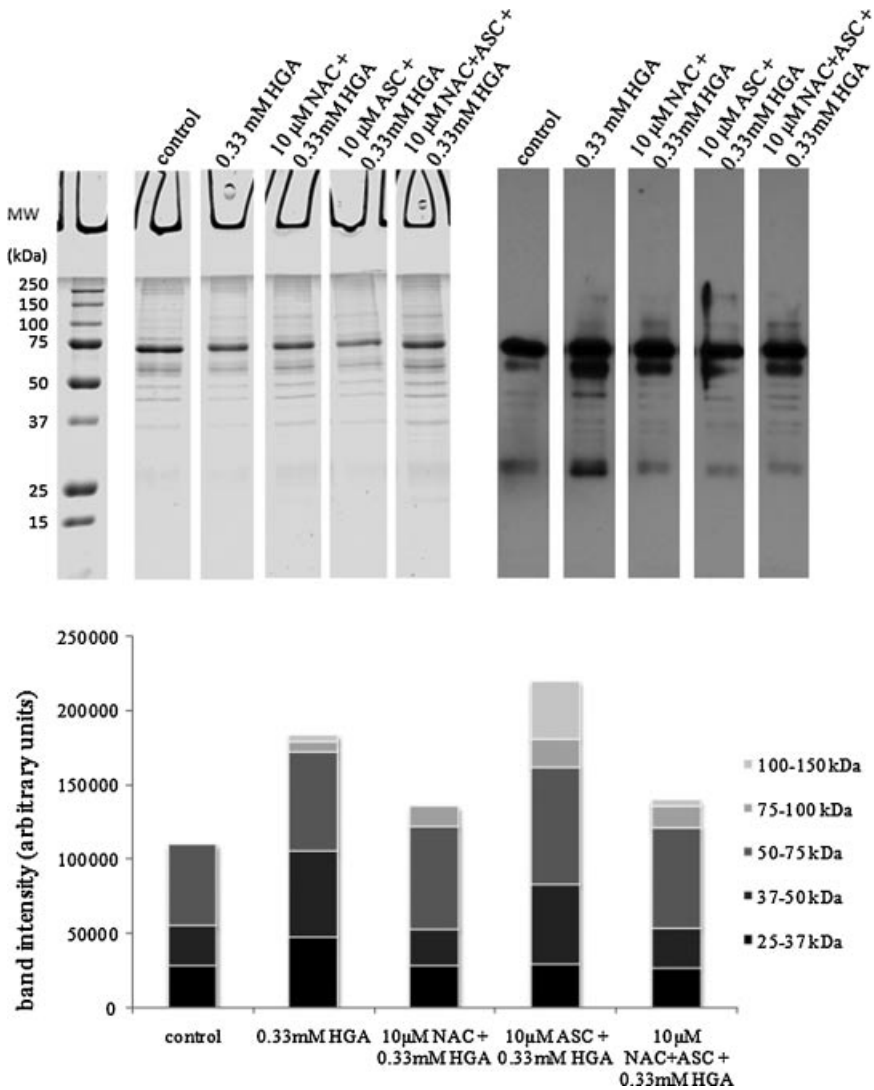

Fig. 4. Coomassie-stained SDS–PAGE (left) and immunoblotting of carbonylated proteins (right) of cells grown in standard conditions, treated with HGA alone, or pre-treated with ASC, NAC, and NAC + ASC before the addition of HGA. Experiments were carried out in triplicate; only representative gels and films are reported.

ochronotic patients (AKU is very rare, 1:250,000–1,000,000 incidence) and in the lack of *in vitro* and *in vivo* models (Kirkpatrick et al., 1984; Angeles et al., 1989). Mice deficient in HGO (hgo−/−) do not normally develop ochronosis, despite the increase in circulating and urinary HGA (Montagutelli et al., 1994; Manning et al., 1999). Progress in understanding the pathogenesis of ochronosis could be achieved by employing a cell model based on human primary cells. The advantage of using non-alkaptonuric human primary cells in the model is that they have physiological characteristics closer to the *in vivo* cell types than those of animals or indeed cell lines.

We have introduced a new *in vitro* model based on primary human non-alkaptonuric articular chondrocytes, grown in HGA-supplemented medium to simulate *in vitro* the conditions of HGA excess leading to the formation of ochronotic pigment *in vivo*. In such a cell model, it was possible to observe substantial HGA-induced intercellular pigment deposition and the presence of pigmented alcapton bodies at the intracellular level and to evaluate the effects induced by different concentrations of HGA on chondrocyte apoptosis and proliferation. To the best of our knowledge, our cell model allowed the first direct observation of *in vitro* biological effects induced by HGA exposure on human chondrocytes at the molecular level. Our AKU experimental model allowed us to investigate cell proliferation, apoptosis, and differentiation. The HGA concentration chosen, 0.33 mM, was within the range of HGA plasma levels in AKU patients (50–400 μM), and was optimal in leading to the formation of ochronotic pigment in a reasonable time with the lowest effect on cell viability. In particular, the cellular exposure to HGA led to: (i) a slight but significant decrease of cell proliferation; (ii) a significant HGA dose-dependent induction of apoptosis without the appearance of necrotic effects; (iii) a substantial decrease in PG release, in contrast to earlier reports (Kirkpatrick et al., 1984).

There is no established cure for AKU. Treatment of arthopathy in AKU is symptomatic, which may be helpful for the early stages of the disease but not for the end stage when total joint replacement is often required. AKU has been reported to disappear following liver transplantation for hepatitis B-related cirrhosis (Kobak et al., 2005), but this is not clinically practicable

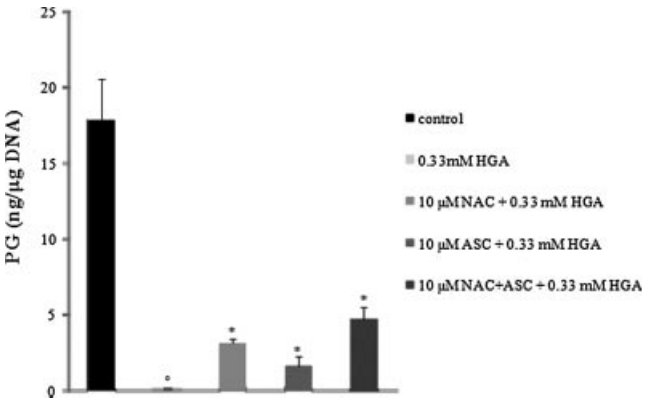

Fig. 5. Proteoglycan release of HGA-treated human articular chondrocytes. Cells were pre-incubated, for 24 h, with NAC and/or ASC at the concentration 10 µM then treated with 0.33 mM HGA for 48 h. Proteoglycan release was assayed by an immunoenzymatic method. The results obtained for the different cultural supernatants were normalized to the DNA content of the culture. Statistical significance compared to the control °$P < 0.05$. Statistical significance compared to 0.33 mM HGA *$P < 0.05$.

for alkaptonuric patients. This disappearance is due to the fact that the bulk of tyrosine catabolism occurs in the liver, with a minor contribution from the kidney (La Du, 2001), this latter playing a critical role in HGA elimination, as witnessed by renal transplantation having a salutary effect in AKU patients with renal failure (Introne et al., 2002). Some treatment modalities have been proposed for AKU (Forslind et al., 1988; Wolff et al., 1989; de Haas et al., 1998) to control HGA levels, but they failed to ameliorate the disease (Kayser et al., 2008).

The herbicide nitisinone (Orfedin) reduces HGA excretion in AKU mice (Suzuki et al., 1999) and humans (Phornphutkul et al., 2002; Suwannarat et al., 2005), but there is no evidence that it prevents or reverses ochronosis, (Keller et al., 2005). A long-term randomized clinical trial in 40 patients (NIH protocol 05-HG-0076) was completed in 2009, which indicated the need of further studies regarding this therapeutic strategy.

The emergence of our experimental model has presented us with an opportunity to undertake pre-clinical testing of potential antioxidant therapies for AKU. As previously reported, ROS are produced during HGA auto-oxidation (Martin and Batkoff, 1987). When ROS levels exceed the antioxidative ability of the chondrocyte, this latter undergoes oxidative stress. As a result, ROS cause damage to DNA and proteins of chondrocytes, leading to apoptosis, functional impairment, and, finally, to cartilage degeneration. Antioxidants should inhibit ochronosis-like pigment formation by maintaining HGA in its reduced hydroquinone form.

From the results derived from our human AKU cell model, we propose a convenient combination of NAC and ASC as a pharmacological intervention in AKU. NAC is an antioxidant already used therapeutically for other purposes. We previously proposed (Chindamo et al., 2003; Lorenzini et al., 2003; Mannoni et al., 2004) NAC for AKU treatment based on three methods of action: (i) acting as a scavenger of ROS, thereby limiting tissue damage; (ii) preventing or delaying HGA oxidation by increasing glutathione (GSH) production; and (iii) by means of a NAC direct neutralization of BQA. Moreover, it has been postulated that cellular GSH may be essential for the function of ASC (Meister, 1992). However, GSH is not transported efficiently into cells (Meister, 1992). In contrast, NAC is readily taken up and de-acetylated inside cells to yield cysteine and subsequently GSH (De Flora et al., 1995). Moreover, NAC and ASC have additive properties and NAC showed the ability to counteract the pro-oxidative effects of ASC (D'Agostini et al., 2000). Recycling of ASC from its oxidized forms is required to maintain intracellular stores and GSH has been shown both *in vitro* and *in vivo* to maintain a reducing milieu in the cell that can reduce dehydroascorbic acid. Most likely, the same function can be provided by NAC, either per se or intracellularly as a precursor of cysteine and GSH. This explains the ability of NAC to prevent the pro-oxidative action of ASC (D'Agostini et al., 2000).

These considerations prompted us to evaluate whether the combination of NAC with ASC could produce beneficial effects in AKU. After investigation on cytotoxicity and pigment deposition induced by HGA on human articular chondrocytes, we demonstrated that a pre-treatment of cells with antioxidants was more effective than co-treatment in reducing cytotoxic effects and pigmentation. Our findings revealed that HGA induced carbonylation of protein and also that NAC and NAC + ASC treatment prevented or delayed this oxidative stress. NAC could increase GSH production and consequently inhibit HGA oxidation to BQA. Clinical trials with ASC confirmed that antioxidants should prevent rather that treat AKU and are particularly efficient if supplemented to infants before the onset of symptoms of ochronosis (Wolff et al., 1989). Moreover, NAC inhibits chondrocyte hypertrophy in endochondral ossification by means of inhibition of apoptosis, angiogenesis, and mineralization (Morita et al., 2007).

We demonstrated that treatment with antioxidant drugs prevented in a dose-dependent manner the growth inhibition associated with HGA exposure. Particularly, the NAC + ASC combined treatment resulted in the greatest influence on cell proliferation. It is noteworthy that even after a long exposure to HGA when it exerts its strongest cytotoxic effect, antioxidants still powerfully counteract its negative effects on cell proliferation. The antioxidant treatment exerts a strong inhibition of HGA-induced cartilage degradation, counteracting: (i) chondrocyte apoptosis; (ii) pigment deposition; (iii) decrease of PG release. Based on our *in vitro* findings, we suggest that prolonged treatment with NAC + ASC could be effective *in vivo* for preventing or delaying the course of ochronotic arthropathy.

Acknowledgments

The authors thank the AKU Society (UK) for its support. We are grateful to Jane Dillon for her comments on the manuscript. Ethics Approval: Approval from the Ethics Committee of the Università degli Studi di Siena.

Literature Cited

Angeles AP, Badger R, Gruber HE, Seegmiller JE. 1989. Chondrocyte growth inhibition induced by homogentisic acid and its partial prevention with ascorbic acid. J Rheumatol 16:4–7.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254.

Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, Orecchia P, Zardi L, Righetti PG. 2004. Blue silver: A very sensitive colloidal Coomassie G-250 staining for proteome analysis. Electrophoresis 25:1327–1333.

Chindamo D, Catenaccio M, Lorenzini S, Selvi E, Molinelli M, Cavallo G, Marcolongo R. 2003. N-acetylcysteine and vitamin E: An in vitro study of their effect on homogentisic acid polymerization. Clin Exp Rheumatol 21:269.

D'Agostini F, Balansky RM, Camoirano A, de Flora S. 2000. Interactions between N-acetylcysteine and ascorbic acid in modulating mutagenesis and carcinogenesis. Int J Cancer 88:702–707.

Dalle-Donne I, Rossi R, Giustarini D, Milzani A, Colombo R. 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin Chim Acta 329:23–38.

De Flora S, Cesarone CF, Balansky RM, Albini A, D'Agostini F, Bennicelli C, Bagnasco M, Camoirano A, Scatolini L, Rovida A, Izzotti A. 1995. Chemopreventive properties and mechanisms of N-Acetylcysteine. The experimental background. J Cell Biochem Suppl 22:33–41.

de Haas V, Carbasius Weber EC, de Klerk JB, Bakker HD, Smit GP, Huijbers WA, Duran M, Poll-The BT. 1998. The success of dietary protein restriction in alkaptonuria patients is age-dependent. J Inherit Metab Dis 21:791–798.

Denizot F, Lang R. 1986. Rapid colorimetric assay for cell growth and survival. Modification to the tetrazolium dye procedure giving improved sensitivity and reliability. J Immunol Methods 22:271–277.
Fernández-Cañón JM, Granadino B, Beltrán-Valero de Bernabé D, Renedo M, Fernández-Ruiz E, Peñalva MA, Rodríguez de Córdoba S. 1996. The molecular basis of alkaptonuria. Nat Genet 14:19–24.
Forslind K, Wollheim FA, Akesson B, Rydholm U. 1988. Alkaptonuria and ochronosis in three siblings. Ascorbic acid treatment monitored by urinary HGA excretion. Clin Exp Rheumatol 6:289–292.
Gaines JJ. 1989. The pathology of alkaptonuric ochronosis. Hum Pathol 20:40–46.
Grigolo B, Roseti L, Neri S, Gobbi P, Jensen P, Major EO, Facchini A. 2002. Human articular chondrocytes immortalized by HPV-16 E6 and E7 genes: Maintenance of differentiated phenotype under defined culture conditions. Osteoarthritis Cartilage 10:879–889.
Helliwell TR, Gallagher JA, Ranganath L. 2008. Alkaptonuria—A review of surgical and autopsy pathology. Histopathology 53:503–512.
Introne WJ, Phornphutkul C, Bernardini I, McLaughlin K, Fitzpatrick D, Gahl WA. 2002. Exacerbation of the ochronosis of alkaptonuria due to renal insufficiency and improvement after renal transplantation. Mol Genet Metab 77:136–142.
Kayser MA, Introne WJ, Gahl WA. 2008. Alkaptonuria. In: Valle D, et al. editors. New online metabolic and molecular basis of inherited disease. New York: McGraw Hill. http://www.ommbid.com.
Keller JM, Macaulay W, Nercessian OA, Jaffe IA. 2005. New developments in ochronosis: Review of the literature. Rheumatol Int 25:81–85.
Kirkpatrick CJ, Mohr W, Mutschler W. 1984. Experimental studies on the pathogenesis of ochronotic arthropathy. Virchows Arch [Cell Pathol] 47:347–360.
Kobak AC, Oder G, Kobak S, Argin M, Inal V. 2005. Ochronotic arthropathy: Disappearance of alkaptonuria after liver transplantation for hepatitis B-related cirrhosis. J Clin Rheumatol 11:323–325.
Kutty MK, Iqbal QM, Teh EC. 1974. Ochronotic arthropathy. An electron microscopic study with a view on pathogenesis. Arch Pathol Lab Med 98:55–57.
La Du BN. 2001. Alkaptonuria. In: Scriver CR, Beaud AL, Sly WS, Valle D, editors. The metabolic & molecular bases of inherited disease, Vol. 2, 8th edition. New York: McGraw-Hill. pp 2109–2123.
La Du BN. 2002. Alkaptonuria. In: Royce PM, Steinmann B, editors. Connective tissue and its heritable disorders, 2nd edition. New York: John Wiley. pp 809–826.
La Du BN, Zannoni VG, Laster L, Seegmiller JE. 1958. The nature of the defect in tyrosine metabolism in alkaptonuria. J Biol Chem 230:251–260.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685.
Lorenzini S, Mannoni A, Selvi E. 2003. Alkaptonuria. N Engl J Med 348:1408.
Manning K, Fernández-Cañón JM, Montagutelli X, Grompe M. 1999. Identification of the mutation in the alkaptonuria mouse model, mutations in brief no. 216. Online. Hum Mutat 13:171.
Mannoni A, Selvi E, Lorenzini S, Giorgi M, Airó P, Cammelli D, Andreotti L, Marcolongo R, Porfirio B. 2004. Alkaptonuria, ochronosis and ochronotic arthropathy. Semin Arthritis Rheum 33:239–248.
Martin JP, Batkoff B. 1987. Homogentisic acid autoxidation and oxygen radical generation: Implications for the etiology of alkaptonuric arthritis. Free Rad Biol Med 3:241–250.
Meister A. 1992. On the antioxidant effects of ascorbic acid and glutathione. Biochem Pharmacol 44:1905–1915.
Montagutelli X, Lalouette A, Coudé M, Kamoun P, Forest M, Guénet JL. 1994. Aku, a mutation of the mouse homologous to human alkaptonuria, maps to chromosome 16. Genomics 19:9–11.
Morita K, Miyamoto T, Fujita N, Kubota Y, Ito K, Takubo K, Miyamoto K, Ninomiya K, Suzuki T, Iwasaki R, Yagi M, Takaishi H, Toyama Y, Suda T. 2007. Reactive oxygen species induce chondrocyte hypertrophy in endochondral ossification. J Exp Med 204:1613–1623.
O'Brien WM, La Du BN, Bunim JJ. 1963. Biochemical, pathologic and clinical aspects of alkaptonuria, ochronosis and ochronotic arthropathy: Review of world literature (1584–1962). Am J Med 34:813–838.
Phornphutkul C, Introne WJ, Perry MB, Bernardini I, Murphey MD, Fitzpatrick DL, Anderson PD, Huizing M, Anikster Y, Gerber LH, Gahl WA. 2002. Natural history of alkaptonuria. New Engl J Med 347:2111–2121.
Selvi E, Manganelli S, Mannoni A, Benucci M, Minacci C, Marcolongo R. 2000. Chronic ochronotic arthritis: Clinical, arthroscopic and pathologic findings. J Rheumatol 27:27–29.
Suwannarat P, O'Brien K, Perry MB, Sebring N, Bernardini I, Kaiser-Kupfer MI, Rubin BI, Tsilou E, Gerber LH, Gahl WA. 2005. Use of nitisinone in patients with alkaptonuria. Metab Clin Exp 54:719–717.
Suzuki Y, Oda K, Yoshikawa Y, Maeda Y, Suzuki T. 1999. A novel therapeutic trial of homogentisic aciduria in a murine model of alkaptonuria. J Hum Genet 44:79–84.
Taylor AM, Wlodarski B, Prior IA, Wilson PJM, Jarvis JC, Ranganth LR, Gallagher JA. 2010. Ultrastructural examination of tissue in a patient with alkaptonuric arthropathy reveals a distinct pattern of binding of ochronotic pigment. Rheumatology. Epub ahead of print.
Vermes I, Haanen C, Steffens-Nakken H, Reutelingsperger C. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labeled Annexin V. J Immunol Methods 184:39–51.
Wolff JA, Barshop B, Nyhan WL, Leslie J, Seegmiller JE, Gruber H, Garst M, Winter S, Michals K, Matalon R. 1989. Effects of ascorbic acid in alkapronuria: Alterations in benzoquinone acetic acid and an ontogenic effect in infancy. Pediatr Res 26:140–144.
Zannoni VG, Malwista SE, La Du BN. 1962. Studies on ochronosis II. Studies on benzoquinoneacetic acid, a probable intermediate in the connective tissue pigmentation in alkaptonuria. Arthritis Rheum 5:547–556.